Rapid Detection and Identification of JC Virus and BK Virus in Human Urine by Using Immunofluorescence Microscopy

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An indirect immunofluorescence method was developed and used to detect urinary excretion of abnormal transitional cells infected with JC virus (JCV) or BK virus (BKV). This method was compared with urinary cytology, electron microscopy, viral culture, and viral serology in groups of immunosuppressed renal transplant recipients and normal controls. The indirect immunofluorescence method detected and identified JCV excretion in four persons, BKV excretion in one person, and both JCV and BKV excretion in eight others. Viral antigen was identified only in the nuclei of cytologically abnormal cells. Of these 13 persons, 8 also had polyoma virions detected in the urine by electron microscopy. With repeated study of sequential urine samples, 30% of transplant recipients and 6% of normal controls were positive by one or more microscopy methods. Serological results confirmed a high incidence of both JCV and BKV multiplication in the immunosuppressed patients. However, serology did not correlate directly with urinary virological findings. Urinary cytology and the indirect immunofluorescence method were rapid and sensitive methods for detecting and identifying urinary excretion of JCV and BKV.

The human polyoma viruses JC virus (JCV) and BK virus (BKV) were first isolated in 1971 (7, 13). Serological studies then demonstrated that most normal persons were infected with these viruses during childhood and adolescence (1, 6, 12, 17). JCV has now been implicated as the etiological agent of progressive multifocal leukoencephalopathy, a demyelinating disorder that occurs in immunodeficient persons (19). BKV has been identified in the urinary tracts of several renal transplant patients with ureteral stenosis (3, 4). Furthermore, urinary excretion of polyoma virus has been found frequently in immunologically altered persons by cytology and electron microscopy (3, 9, 14, 15). The excreted virus has sometimes been identified as BKV (3, 4, 8, 15) or JCV (14) by viral culture or by immune electron microscopy (4). However, culture methods for these viruses have been time-consuming, and relatively few isolations have been possible (2–4, 15). Similarly, immune electron microscopy has sometimes been difficult when the excreted virions were coated with antibody (15).

Immunofluorescence microscopy (IFM) has been used to rapidly identify JCV in virus-infected brain (19). Therefore, we tested the suitability of IFM for the detection and identification of JCV or BKV in exfoliated, transitional cells in the urine. These cells line the urinary tract, including the renal pelvis, ureter, bladder, and proximal urethra (5). Our objective was to compare IFM with urinary cytology, electron microscopy, viral culture, and viral serology for the detection of JCV or BKV excretion.

MATERIALS AND METHODS

Subjects. Sixty-one immunosuppressed renal transplant recipients and 16 normal persons consented to give urine and serum samples for polyoma viral studies. These individuals were repeatedly tested during a 2-year period. The two groups were similar in average age, sex ratio, and time spanned by the collected samples. In addition, 31 normal persons had single urine samples tested.

Indirect IFM. A rabbit antiserum was prepared by injecting JCV subcutaneously into the shoulders and into each hind footpad. Each of these injections contained 5,000 hemagglutination units of virus in Freund complete adjuvant. After 3 weeks, 180,000 hemagglutination units suspended in physiological saline was injected by the peritoneal route. Serum was collected 2 weeks after the intraperitoneal injection and was found to have a hemagglutination inhibition (HI) titer of 1:1,024,000 against 8 hemagglutination units of JCV. At a dilution of 1:4 in phosphate-buffered saline, this serum gave a strong reaction in the indirect immunofluorescence test against JCV, BKV, and simian virus 40 and was used as a trivalent screening serum. Monospecific rabbit antisera were prepared by injecting 10,000 hemagglutination units of virus (either JCV or BKV) intravenously and collecting serum 10 to 12
days later. Such sera had HI titers of about 1:5,192 against the virus used to stimulate antibody production. Those sera that lacked cross-reacting antibody by the HI test were adsorbed with primary human fetal brain cells to minimize nonspecific staining. Antibody specificity was then confirmed in indirect immunofluorescence tests by using cells infected with JCV, BKV, simian virus 40, and human cytomegalovirus and a number of uninfected human and animal cells (both transformed and diploid).

Fluorocarbon-masked, multwell microscope slides (10 wells, 7-mm diameter; Cell Line Associates, Minotola, N.J.) were cleaned, rinsed, digested in warm 1% gelatin in distilled water, air dried, and stored at room temperature.

Fresh urine specimens were collected and stored (4°C) for no longer than 8 h. The pH was adjusted to neutrality if crystals were present with 7.5% NaHCO₃, 1 N HCl, and pH paper. A 10-ml sample was centrifuged for 10 min at 1,500 × g, and the sediment was suspended in a few drops of urine. One drop of urine sediment was supplied to each well; slides were air dried, fixed in fresh dehydrated acetone for 15 min at room temperature, and stored at -20°C until examined.

Multwell slides were thawed and tested for viral intranuclear antigen by adding monospecific anti-JCV or anti-BKV antiserum or the trivalent screening antisem. Slides were incubated in moist chambers for 30 min at room temperature, washed twice in phosphate-buffered saline, incubated with commercial fluorescein-conjugated, goat anti-rabbit globulin (Antibodies Inc., Davis, Calif.) for 30 min at room temperature, and then washed twice again in phosphate-buffered saline. Wash times of 5 and 20 min produced optimal results. Underwashing left a nonspecific haze which made nuclear fluorescence difficult to detect, whereas overwashing produced cell loss and false-negative results. Cover glasses were mounted on the slides (phosphate-buffered saline–glycerin, 1:10, pH 7.0), and slides were kept at 4°C until examined.

Abnormal cells with nuclear fluorescence were never numerous in any sediment. Therefore, each antiserum was usually tested on four wells to enhance the detection of antigen-containing cells. Any slide with one or more clearly positive cells was scored as positive. Faint, nonspecific cytoplasmic fluorescence made cell outlines visible, so counterstaining was unnecessary.

Cytology. One drop of urine sediment was also applied to each of two microscope slides. Slides were air dried, fixed in 95% ethyl alcohol, and stained by standard Papanicolaou and hematoxylin-eosin methods. The whole sediment on each slide was examined for abnormal, inclusion-bearing transitional cells, previously associated with urinary polyoma virus excretion (2, 3). These cells were 15 to 35 μm in diameter, had an increased nuclear-to-cytoplasmic ratio, and contained dark basophilic intranuclear inclusions and a thickened nuclear membrane. The inclusions have been shown by electron microscopy to be crystalline arrays of polyoma virions (7, 14).

Initially, cytocentrifuge and membrane filter (Millipore Corp., Bedford, Mass.) preparations of urine sediment were also examined. However, these methods neither enhanced the yield of abnormal transitional cells nor greatly improved cytological detail. Therefore, the clinical centrifuge method was used routinely.

Electron microscopy. Ten milliliters of fresh urine was centrifuged at 100,000 × g for 1 h. Pellets were either examined immediately or suspended in 1 ml of Eagle minimal essential medium with 2% fetal calf serum, frozen at -80°C, and examined later. Ten milliliters of distilled water was added to the fresh or thawed urine pellet, and samples were recentrifuged at 100,000 × g for 1 h. A portion of this second pellet was taken, using a sterile loop, and was placed on a Formvar-carbon-coated, 300-mesh copper grid. Excess liquid was blotted off with a piece of filter paper, a loopful of 2% (wt/vol) phosphotungstic acid (pH 6.0) was added, and the grid was blotted a second time. Grids were then examined in a Philips 300 electron microscope at 80 to 100 kV and X71,000 magnification for 10 to 20 min each. Positive JCV or BKV control grids were included for a direct comparison with virus particles excreted in the urine.

Cultures. Thawed urine sediments were sonicated at 100 W for 2 min with a Branson W-350 Sonifier. One-tenth milliliter per tube was inoculated onto spongiosblast-rich, primary human fetal brain monolayers grown on cover slips in Leighton tubes. After 2 h of incubation at 37°C, Eagle minimal essential medium with 2% fetal calf serum was added, and cultures were incubated for 4 weeks. The medium was changed, and tubes were inspected for cytopathic effect biweekly (11). After 4 weeks, monolayers were frozen and thawed, sonicated, and treated with neuraminidase (Microbiological Associates, Walkersville, Md.), and samples were passed to fresh cell monolayers. After two to three such serial blind passages, the cultures were examined for intranuclear polyoma viral antigen by fluorescence microscopy. Several samples were also cultured for 4 weeks in human embryonic kidney monolayers, which support BKV growth (10, 16).

Serology. Two or more sera from each subject were titrated with both JCV and BKV by HI, using human type O erythrocytes and standard microtiter methods (12). Fourfold or greater antibody titer rises were considered indicative of active infection.

RESULTS

HI serology detected a 41% incidence of polyoma virus infection in the 61 immunosuppressed renal transplant recipients (Table 1). Eighteen (30%) had fourfold or greater rises against JCV, whereas 11 (18%) had rises against BKV. In contrast, only one normal person (6%) had a titer rise against BKV.

Positive urine microscopy results (Fig. 1) were found in 30% of the 61 immunosuppressed renal transplant recipients. Eighteen (30%) were positive by one or more methods, 15 (25%) were positive by two or more methods, and 9 (15%) were positive by all three methods. In contrast, only 1 of 16 normal persons (6%) was positive by these methods (cytology and IFM showed JCV
**Table 1. IFM compared with other methods for detecting polyoma virus activity in 61 renal transplant patients**

<table>
<thead>
<tr>
<th>Other method</th>
<th>Positive (n = 17) by IFM</th>
<th>Negative (n = 44) by IFM</th>
<th>No. positive/ no. tested</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
<td>No. negative</td>
<td>No. positive</td>
<td>No. negative</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cytology</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>43</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>8</td>
<td>9</td>
<td>1</td>
<td>36</td>
</tr>
<tr>
<td>Culture*c</td>
<td>0</td>
<td>7</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Serology*e</td>
<td>7</td>
<td>10</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>

* By IFM, 17/61.
* By IFM, 28%.
* Nineteen samples from seven persons tested.
* ND, Not done.
* Fourfold or greater HI antibody titer against either JCV or BKV.

**Fig. 1.** Typical microscopy findings in urine sediments from polyoma virus urinary excretors. (A) Polyoma virus nuclear antigen detected in an abnormal transitional cell by using a trivalent rabbit antiserum against JCV, BKV, and simian virus 40. Similar antigen was demonstrable in other cells by using monovalent anti-JCV or anti-BKV serum. (B) Abnormal transitional cell with a basophilic intranuclear inclusion and thickened nuclear membrane. A normal squamous cell is shown below for comparison. Hematoxylin-eosin stain. (C) Polyoma virus particles in urine sediments from three renal transplant recipients. Their urine samples were also positive by cytology and IFM. Virus particles were negatively stained with neutral 2% (wt/vol) phosphotungstic acid.
excretion, whereas HI antibody titers against JCV and BKV remained at 512 for 36 months.

The results of the three microscopy methods correlated with each other (Table 1). IFM-positive cells were usually found in samples from patients excreting cytologically abnormal cells, which have been previously associated with polyoma virus urinary excretion (2, 3). By IFM, viral antigen was identified only in the nuclei of these cells (Fig. 1) and not in epithelial cells, leukocytes, or normal transitional cells. By IFM, only JCV nuclear antigen was found in urine samples from three patients (and from the one normal person), and only BKV nuclear antigen was found in samples from one patient. Both JCV and BKV antigens were found in samples from eight patients. In five patients, both JCV and BKV were detected simultaneously in the same sample. In three patients, JCV and BKV were detected in separate samples by repeated testing. Most of the above persons excreted cytologically abnormal cells on several occasions over a long period of time. In contrast, five patients had polyoma virus nuclear antigen detected only by the high-titered screening antisera. Antigen was not further identified by the weaker, monospecific antisera. These patients rarely had cytologically abnormal cells detected in urine samples, even with repeated study.

Polyoma virus particles were typically identified by electron microscopy in urine in which abnormal cells and nuclear antigen were repeatedly seen (Table 1). A prolonged search of several sample grids was often necessary before virus particles could be identified. No sample had more than a few particles detected, and capsids often appeared empty or antibody coated (Fig. 1).

The three microscopy methods were tested further by studying single urine samples from 31 additional normal persons. One person (3%) had a few polyoma virus particles noted by electron microscopy. All samples were negative by cytology and IFM.

Sequential viral studies in three immunosuppressed patients are shown in Table 2. In these patients, serum antibody titer changes seemed to correspond to the urinary microscopy findings. For example, patient no. 1 had a significant titer rise against JCV, and abnormal transitional cells, JCV nuclear antigen, and polyoma virus particles were demonstrable in her urine. Patient no. 2 seroconverted to both JCV and BKV, and abnormal transitional cells, polyoma virus particles, and both JCV and BKV nuclear antigens were found in the urine. Patient no. 3 had a

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Abnormal transitional cells</th>
<th>Polyoma virus nuclear antigen</th>
<th>Polyoma virus particles</th>
<th>Culture results</th>
<th>HI antibody titer</th>
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<td>No. 1, 40-year-old female</td>
<td>11/75 (Transplant)</td>
<td>7/76 (+) 8/76 (+) 9/76 (-) 11/76 (-) 1/77 (-) 3/77 (+) 6/77 (-)</td>
<td>JCV 32</td>
<td>BKV 512</td>
<td></td>
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<tr>
<td>No. 2, 4-year-old male</td>
<td>1/77 (Transplant)</td>
<td>3/77 (-) 4/77 (+) 5/77 (+) 7/77 (+) 1/78 (-)</td>
<td>JCV 8</td>
<td>BKV 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 3, 56-year-old female</td>
<td>7/75 (Transplant)</td>
<td>7/76 (-) 10/76 (-) 12/76 (-) 2/77 (+) 3/77 (+) 4/77 (+) 6/77 (+)</td>
<td>JCV 256</td>
<td>BKV 256</td>
<td></td>
<td></td>
</tr>
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</table>

* Trivalent antiserum against JCV, BKV, and simian virus 40.

b NT, Not tested.
gradually rising titer against BKV, and all three microscopy methods were repeatedly positive. Although titer changes in these patients did correspond to the urinary microscopy findings, this was not the general case. Equal percentages of both virus excretors and nonexcretors had titer increases (Table 1). In addition, urine cultures for JCV and BKV were negative in 19 samples obtained from seven patients. All of these patients had positive urine cytology, IFM, and electron microscopy on several occasions (Tables 1 and 2).

DISCUSSION

We found IFM to be a rapid and useful method for specifically identifying urinary excretion of JCV or BKV. IFM detected JCV excretion in 11 patients and 1 normal person and BKV excretion in 9 patients. Five additional patients had polyoma virus nuclear antigen detected only with the high-titered screening antiserum. Presumably, the lower-titered nonspecific antisera would also work in these sediments if more cytologically abnormal cells were present. We identified polyoma viral antigen only in the nuclei of these cells (Fig. 1), which have previously been associated with JCV or BKV urinary excretion (2, 3, 14).

Prior studies have reported a somewhat higher incidence of abnormal urine cytology (3) or electron microscopic identification of polyoma virus particles (9) in the urine of immunosuppressed transplant recipients. Some have also identified BKV in these urines by using viral culture, although the yield has been relatively low (3, 4, 8, 15, 18). Our urine culture results were negative (Table 1), and repeated study of sequential urine samples, using cytology, electron microscopy, and IFM, was needed to get the clearest picture of JCV or BKV excretion (Table 2). However, our transplant recipients received less exogenous immunosuppressant than did patients in most of the prior studies. Also, our electron microscopic results suggested that few virus particles were present in our urine samples, and most seemed broken or antibody coated (Fig. 1).

Both the IFM results and the HI serological results confirmed a high incidence of both JCV and BKV multiplication in these patients. However, the serological results did not predictably correlate with urinary polyoma virus excretion (Table 1). IFM is a useful complementary method for the determination of papovavirus excretion. Techniques for concentrating abnormal, virus-infected transitional cells from urine (e.g., differential sedimentation or flow cytometry) might make IFM even more attractive for rapid, specific polyoma virus identification.

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